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RESEARCH

Identification of Molecular Markers Associated with Root-Knot Nematode Resistance in Upland Cotton

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ABSTRACT

Cotton breeding for resistance to root-knot nematode (RKN) [*Meloidogyne incognita* (Kofoid and White) Chitwood] is hindered by the lack of convenient and reliable screening methods for resistant plants. The identification of molecular markers closely linked to RKN resistance will facilitate the development of RKN resistant cultivars through marker-assisted selection (MAS). Our objective was to identify and develop new DNA markers that are associated with RKN resistance in cotton. Using three pairs of near-isogenic (NIL) resistant (R) and susceptible (S) lines, two AFLP markers, two RAPD markers, and three RGA markers were identified to be polymorphic between the NIL-R and NIL-S lines. One RAPD marker was converted into a sequence-tagged site (STS) marker. In an F_2 population of 'ST 474' × 'Auburn 634 RNR', the two RAPD markers and the STS marker were mapped to the same linkage group containing several markers that were previously reported to be linked with the RKN resistance gene *rkn1* on chromosome 11 in 'Acala NemX'. All these markers were found to be associated with a major RKN resistance gene, presumably Mi_2 in the resistant line Auburn 634 RNR, suggesting that *rkn1* and Mi_2 are either allelic or closely linked. In addition, no susceptible recombinants were found in a resistance screen of 200 F_2 plants from the cross Acala NemX × Auburn 634 RNR. The utility of the two RAPD markers and the converted STS marker were evaluated using 23 R and 8 S germplasm lines. The RAPD and STS markers, along with other previously reported markers associated with RKN resistance will be useful in germplasm screening, MAS for RKN resistance, and map-based cloning for RKN resistance genes.

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Abbreviations: AFLP, amplified fragment length polymorphism; CAP, cleaved amplified polymorphism; MAS, marker-assisted selection; NIL, near isogenic line; RAPD, random amplified polymorphic DNA; RGA, resistance gene analogue; RKN, root-knot nematode; SSR, simple sequence repeat; STS, sequence-tagged site.

Upland cotton (*Gossypium hirsutum* L.), one of the most economically important crops in the world, provides raw material for the textile and oil industry. In the USA in 2004, the estimated total cotton yield loss due to diseases was 10.93% (Blasingame and Patel, 2005). Among various cotton diseases, the southern root-knot nematode (RKN), *Meloidogyne incognita* (Kofoid and White) Chitwood race 3, has become a widespread, destructive pest throughout the Cotton Belt (Kirkpatrick and Rothrock, 2001). The nematode forms galls or 'knots' on the roots of cotton, and limits the ability of plants to uptake and transport water and nutrients (Bridge, 1992). Nematode infection also may increase the susceptibility to Fusarium wilt (Minton and Minton, 1966). Yield losses in cotton attributed to RKN damage in 2004 across the U. S. Cotton Belt were 545 728 bales, about 2.49% (highest among cotton diseases) of the total U. S. cotton production (Blasingame and Patel, 2005).

Use of resistant cultivars is considered to be an effective, economic, and environmentally sustainable strategy for managing RKN.

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Although moderate to high levels of resistance to RKN are reported in primitive accessions of upland cotton (Shepherd, 1974a, 1983; Robinson and Percival, 1997; Robinson et al., 2004), and breeding lines (Shepherd 1974a, b; 1982; Shepherd et al., 1989, 1996; Robinson and Percival, 1997), only four commercial cultivars exhibit moderate resistance with limited planting acreage: Stoneville LA 887 and Paymaster 1560 developed from LA 434 RKR which was derived from Clevewilt 6-8 (Jones et al., 1991), Stoneville 5599BR derived from LA 887, and CPCSD 'Acala NemX' developed from an unknown resistance source (Robinson and Percival, 1997; Robinson et al., 2001). Developing highly resistant cultivars will increase the choice of cultivar adaptation and seed availability to facilitate their widespread application.

Molecular marker-assisted selection (MAS) has provided potential for efficient development of disease- and pest-resistant plants. A successful breeding program for nematode resistance depends largely on the identification of effective resistance sources and the inheritance of the resistance. In cotton, RKN resistance genes have been reported in germplasm lines, such as Mi_1 and Mi_2 in an 'Auburn 623 RNR' derived line, M-315 RNR, Mi_1 in a day-neutral converted line M-78 RNR (McPherson et al., 2004), and *rkn1* in Acala NemX (Wang et al., 2006a,b; Wang and Roberts, 2006), although the genetic relationships of RKN resistance from different cotton germplasm lines are not known (Zhou et al., 1999; Bezawada et al., 2003; McPherson et al., 2004; Zhang et al., 2004, 2006b, 2007b). Identification of reliable molecular markers and confirmation of their practical usefulness are highly desirable for RKN resistance research. Bezawada et al. (2003) reported two polymorphic simple sequence repeat (SSR) markers that could explain less than 10% of the variation for RKN resistance in an F_2 population from a cross between Clevewilt 6 and ST 213, but the relationship between the SSR markers and the RKN resistance trait was not confirmed (Zhang et al., 2004). One of the reasons could be that RKN resistance screening is not reliable on an individual plant basis (Zhang et al., 2006a). Recently, by using degenerate disease resistance (R) gene primers to compare susceptible and resistant near-isogenic lines (NIL), Hinchliffe et al. (2005) isolated 165 resistance gene analog (RGA) nucleotide sequences. The putative association of these RGA nucleotide sequences with RKN resistance was also investigated. However, no reliable molecular markers tightly linked to cotton RKN resistance in Auburn 623 RNR had been reported until very recently. Two SSR markers amplified by primers BNL1231 and CIR316 and one cleaved amplified polymorphic sequence (CAPS) marker (GHACC1) converted from an AFLP marker were found to be tightly linked to the resistance gene *rkn1* in Acala NemX (Wang et al., 2006b; Wang and Roberts, 2006). The *rkn1*-linked markers provide the opportunity to investigate the relationship of *rkn1* to the Auburn and other RKN resistance sources. The association between the SSR markers BNL1231 or CIR316 on the long arm of Chromosome 11 and RKN resistance in segregating populations involving the Auburn 623 RNR-derived resistant lines has been confirmed recently by Shen et al. (2006) and Ynturi et al. (2006). Ynturi et al. (2006) further demonstrated that the chromosome region anchored by SSR markers BNL3661-186 and BNL545-118 located on the short arm of Chromosome 14 is also significantly associated with RKN resistance. Shen et al. (2006) detected a minor QTL on Chromosome 7 affecting RKN resistance.

In this study, we used amplified fragment length polymorphism (AFLP) (Vos et al., 1995), random amplified polymorphic DNA (RAPD) (Williams et al., 1990), and resistance gene analogs (RGA) (Hinchliffe et al., 2005; Zhang et al., 2007a) to search for DNA marker(s) associated with the RKN resistance based on comparisons between three resistant near-isogenic breeding lines (NIL-R) and their susceptible parents. The inclusion of RGA markers explores the possibility of targeting potential candidate R gene(s) associated with disease resistance by using degenerate primers designed mostly from the predominant nucleotide-binding sites (NBS) and leucine-repeat regions (LRR) of well characterized R-gene products (Leister et al., 1996; Yu et al., 1996; Chen et al., 1998; Madsen et al., 2003; Tan et al., 2003). The resistance gene resource of these NIL-R originated from the highly resistant breeding line Auburn 623 RNR developed from a cross between Clevewilt 6 and Wild Mexico Jack Jones (Shepherd, 1974b). Auburn 623 RNR and its derived lines have the highest level of RKN resistance known in upland cotton germplasm (McPherson et al., 1995; Shepherd et al., 1996). The high level of RKN resistance of these NIL-R has been evaluated in multiple tests and confirmed on a population basis. The NIL-R were developed through two generations of backcrossing and many generations of selfing, and only DNA fragments linked with the resistance genes should have been transferred into the resistant NIL. These resistant lines and their recurrent parents have similar genetic backgrounds and could be considered NIL (Monforte and Tanksley, 2000). In comparison with their susceptible recurrent parents, consistent presence or absence of DNA markers in the NIL-R under different backgrounds would be an indication that these markers are linked to the RKN resistance.

The objectives of this research were to (i) identify and develop molecular markers associated with the RKN resistance in upland cotton; (ii) test the feasibility of these markers with a wide range of RKN resistant and susceptible cotton germplasm; and (iii) validate the identified markers in a population segregating for RKN resistance.

MATERIALS AND METHODS

Plant Materials and Genomic DNA Isolation

For initial screening of RKN resistance-associated polymorphisms, three RKN resistant lines (M-240 RNR, M-249 RNR, and M-120 RNR) and their respective susceptible parents ['Deltapine

(DP) 61', 'Stoneville (ST) 213', and 'Coker 201'] were compared (Shepherd et al., 1989; 1996). Their RKN-resistance donor was 'Auburn 634 RNR' (Shepherd, 1982), which was derived from a cross and backcross between 'Auburn 56' as a recurrent parent and Auburn 623 RNR as the donor for RKN resistance (Shepherd, 1974b). To test the feasibility of molecular markers obtained from the comparison, an F_2 population was made from a cross between 'ST 474' and Auburn 634 RNR. Additional susceptible and resistant breeding lines (Table 1) were collected and grown in the greenhouse. The F_2 population together with the parental lines was evaluated in the greenhouse for RKN resistance using a galling index ranging from 0 to 6 (modified from Zhang et al., 2006a), where ratings 0, 1, 2, and 3 were considered resistant, and 4, 5, and 6 susceptible according to the responses of both parents (average ratings for ST 474 and Auburn 634 RNR were 3.0 and 1.0, respectively). An F_2 population of 200 individuals from the cross Acala NemX × Auburn 634 RNR, plus five plants of each parent, were phenotyped for resistance in the greenhouse by inoculating each 3-wk-old plant with 40 000 *M. incognita* eggs and scoring roots for galling symptoms 60 d after inoculation. Genomic DNA was isolated from leaf tissue of each genotype following the established mini-prep protocol (Zhang and Stewart, 2000). DNA quantity was measured by a TD-360 Mini-Fluorometer (Turner Designs, Sunnyvale, CA) with Hoechest dye and checked for integrity by resolving on a 0.8% agarose gel.

AFLP Analysis

AFLP procedures were performed as outlined by Vos et al. (1995) with minor modifications (Zhang et al., 2005). The genomic DNA (100 ng/μL) digestion and adaptor ligation were conducted in a 10 μL reaction at 37°C for 3 h with the following reagents: 0.2μL of T4 DNA Ligase (400U/μL), 1 μL of 10 × Ligase buffer, 1 μL of NaCl (0.5 *M*), 0.5 μL BSA (1 mg/mL), 1 μL of *Mse*I adaptor (25 μ*M*), 1 μL of *Eco*RI adaptor (5 μ*M*), 0.5 μL of *Mse*I enzyme (10 U/μL), 0.25 μL of *Eco*RI enzyme (20 U/μL), and 4.55μL H_2O.

Table 1. Presence (+) or absence (–) of two RAPD markers UBC693–420 and UBC781–1000, UBC781-STS, UCR-STS, and CIR316 linked to RKN in three pairs of cotton NIL and cultivars/breeding lines resistant (R) or susceptible (S) to RKN. AB: Auburn; DP: Deltapine; CK: Coker; ST: Stoneville.

Genotype	PI No.	Pedigree	Reaction to RKN	UBC 693-420	UBC 781-1000	UBC 781-964 STS	UCR-STS	CIR 316	Group
DP 61			S	–	–	–	–	–	3
M-240	592511	AB 634 RNR/DP61	R	+	+	+	+	+	1
CK 201			S	–	–	–	–	–	3
M-120	592509	AB 634 RNR/CK201	R	+	+	+	+	+	1
ST 213			S	–	–	–	–	–	3
M-249	592512	AB 634 RNR/ST213	R	+	+	+	+	+	1
AB 623 RNR	SA 1492		R	+	+	+	+	+	1
AB 634 RNR	GP 166	AB 623 RNR/AB 56	R	+	+	+	+	+	1
Clevewilt 6	SA 245		R	–	–	–	+	+	3
M-19 RNR	517931	*Richmondii*/ DP 16	R	+	–	–	+	+	2
M-22 RNR	517932	*Latifolium*/ DP 16	R	+	–	–	+	–	2
M-25 RNR	517933	*Punctatum*/ DP 16	R	+	–	–	+	–	2
M-26 RNR	517934	*Punctatum*/ DP 16	R	+	–	–	+	–	2
M-27 RNR	517927	*Punctatum*/ DP 16	R	+	–	–	+	–	2
M-28 RNR	517928	*Punctatum*/ DP 16	R	+	+	+	+	–	1
M-70 RNR	517930	*Latifolium*/ DP 16	R	+	–	–	+	+	2
M-75 RNR	517929	*Latifolium*/ DP 16	R	+	–	–	+	+	2
M-78 RNR	517930	*Latifolium*/ DP 16	R	+	+	+	+	+	1
M-188 RNR	517936	*Latifolium*/ DP 16	R	+	+	+	+	–	1
M-331 RNR	592515	AB 634 RNR/AB56	R	+	+	+	+	+	1
M-487 RNR	517937	*Punctatum*/ DP 16	R	+	+	+	+	+	1
M-495 RNR	517938	*Punctatum*/ DP 16	R	+	–	–	+	–	2
M-725 RNR	592516	AB 634 RNR/CK310	R	+	–	–	+	+	2
GA 96-211	633019	GA77-27/'PD-3'//GA88-92/3/M-240-RNR/4/M-120-RNR/5/'LA887'	R	+	+	+	+	+	1
Wild Mexican Jack Jones	TX 2516		R	+	–	–	+	–	2
Pima 3-79			S	–	–	–	+	–	3
NM 24016			S	–	–	–			3
Pima 32			S	–	–	–	+	–	3
K239			S	–	–	–	+	–	3
K240			S	–	–	–	+	–	3
Acala NemX			R	+	+	+	+	+	1

Following the ligation of the *Eco*RI and *Mse*I adaptors to the restricted DNA, two primers with a single selective nucleotide extension (forward *Eco*RI-PSA E: 5'-GACTGCGTACCAATTCA; reverse *Mse*I-PSA M: 5'-GATGAGTCCTGAGTAAC) were used in the pre-selection amplification (PSA). To survey for polymorphism, the subsequent PCR amplification procedure using *Eco*RI PSA primer plus 2 nucleotides in combination with *Mse*I PSA primer plus 2 nucleotides was performed with a "touchdown" program as follows: initial denaturing at 94°C for 12 min; ten cycles of 94°C for 20 s, 66°C for 30 s, and 72°C for 2 min, each with 1°C lowering of annealing temperature, followed by 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, and a final extension at 60°C for 30 min after the last PCR cycle. The PCR products were loaded onto a 30-cm 5% polyacrylamide gel made from 5% Long Ranger acrylamide solution (Cambrex Bio Science Rockland, Inc., Rockland, ME), 1×TBE, 0.025% ammonium persulfate, and 0.1% TEMED. The polyacrylamide gel was run at 50–60 W for 2–2.5 h, and viewed by silver-staining. Gel photograph was recorded by using zoom digital camera Kodak EDAS 290 (Eastman Kodak Company, New Haven, CT). Using *Eco*RI primers labeled with a fluorescent dye WellRed D4 (Beckman-Coulter Inc., Fullerton, CA), AFLP products of pooled DNA sample from three resistant lines and three susceptible lines also were analyzed using the CEQ 8000 Genetic Analysis System (Beckman-Coulter Inc., Fullerton, CA) in initial primer screening.

RGA Analysis

Eight degenerate primer pairs designed according to the conserved motifs of several cloned plant R genes were used (Hinchliffe et al., 2005). These degenerate primer pairs were originally reported by Leister et al. (1996), Yu et al. (1996), Feuillet et al. (1997), and Chen et al. (1998). We performed PCR reactions in 20μL volumes with the following concentrations: 1×PCR buffer (10mM Tris-HCl, 50mM KCl, pH 8.3), 2ng/μL genomic DNA template, 0.2mM deoxy-nucleotide triphophates (dNTPs), 2.5mM $MgCl_2$, 2μM of each primer, and 0.025U/μL of Taq DNA polymerase. Thermal cycling conditions were initial denaturing at 94°C for 4 min followed by 6 cycles at 94°C for 30 s, 35°C for 30 s, and 72°C for 60 s to increase the amplification from low Tm RGA primers, then 40 cycles at 94°C for 60 s, 43°C for 45 s, and 72°C for 90 s with a final extension at 72°C for 7 min. To separate the potential heterogeneous DNA fragments efficiently, high resolution 5% polyacrylamide gels as described previously were used. The polyacrylamide gel method was based on Chen et al. (1998) without using urea.

RAPD Analysis

RAPD reactions were conducted in a total volume of 25 μL, using 20 ng of cotton DNA, 1×PCR Buffer (10mM Tris-HCl, 50mM KCl, pH 8.3), 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 0.2 μM of 10-mer primers (synthesized by Sigma-Genosys, Woodlands, TX), and 1.0 unit of Taq (Zhang and Stewart. 2000). The amplification profile consisted of an initial period of denaturation at 94°C for 2 min, followed by 45 cycles of 94°C for 15 s, 40°C for 30 s, and 72°C for 90 s. The PCR amplifications were completed by a final extension at 72°C for 7 min. 1% agarose gel was used in initial screening of polymorphic RAPD bands. Putative polymorphic RAPD markers were analyzed further on a 5% polyacrylamide gel as described earlier and excised for cloning. All PCR reactions in this study were performed in a PE Applied Biosystems GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA).

Sequencing of PCR Products

Potential polymorphic AFLP, RAPD, and RGA markers were isolated from polyacryamide gels, reamplified, and cloned into pGEM-T Easy vector following the manufacturer's manual (Promega Corporation, Madison, WI). Competent *Escherichia coli* DH5α cells were generated and transformed. For each *E. coli* transformation, five to ten white colonies were picked for sequencing. The recombinant plasmid DNA was sequenced using a LI-COR DNA sequencer. For sequence quality checking, all clones were sequenced in two directions by using M13 forward and M13 reverse primers. Sequences were trimmed for correct insertion by comparing with pGEM-T vector using NCBI (http://www.ncbi.nlm.nih.gov/BLAST; verified 27 March 2007) alignments program. Alignment of the DNA sequence from multiple clones was made by means of the ClustalW program (available at http://www.ebi.ac.uk/clustalw; verified 27 March 2007). Based on the sequence information, sequence specific primers were designed for the development of sequence-tagged site (STS) markers associated with the RKN resistance. Primers were designed for the flanking regions of the cloned fragments using a web-based software, "Primer3" (http://primer3.sourceforge.net; verified 27 March 2007), and the primers were synthesized by Sigma-Genosys.

Survey of RAPD, SSR, CAPS, and STS Markers in a Segregating Population and Diverse Cotton Germplasm

Genomic DNA isolated from an F_2 population (90 plants) of ST 474 (susceptible) × Auburn 634 RNR (resistant), and susceptible and resistant cotton genotypes (Table 1) were used as PCR templates to test the usefulness of six markers including two RAPD markers (UBC693-420 and UBC781-1000) and one STS marker (UBC781-964STS) identified in the current research, and three markers identified previously for *rkn1* in Acala NemX. PCR reactions for the STS marker were performed in a 10 μL reaction with the following concentrations: 0.5 μM primers, 2ng/μL genomic DNA template, 2.5 m*M* $MgCl_2$, 0.2 m*M* each dNTPs, and 0.05U/μL Taq DNA polymerase. Thermal cycling conditions were 5 min at 94°C for initial denaturation followed by 35 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 45 s. A final extension was performed at 72°C for 5 min. One percent agarose gel and ethidium bromide staining were used to check the PCR products. Two SSR primer pairs (BNL1231: 5'-TAATAAAAGGGAAAGGAAAGAGTT and 5'-TATGGCTCTAGAATATTCCCTCG and CIR316: 5'-CTTACAGGCACTACCACC and 5'-CCCTTTCTGGCGACTT) and one CAPS marker (GHACC1) primer pair (E-AAG/M-CCG-300F: 5'-AAGTTTAGTCAACTTCTAAA; E-AAG/M-CCG-300R: 5'-CCGGTGGGTTATTGCCTGAC) that amplify markers linked with RKN resistance gene *rkn1* in Acala NemX, were also tested based on Wang et al. (2006b), and Wang and Roberts (2006).

A linkage map comprising the newly identified and previously reported markers and RKN resistance was constructed

using MAPMAKER/EXP 3.0 (Lander et al., 1987). A LOD score of 3.0 and recombination fraction of 0.4 were used to establish the linkage. Recombination frequencies were converted into centiMorgans (cM) using the Kosambi mapping function.

RESULTS

Putative Polymorphic AFLP, RAPD, and RGA Markers Associated with RKN Resistance

Initially 64 AFLP primer pairs from 8 *Eco*RI selective primers in combination with 8 *Mse*I selective primers were tested using pooled resistant and susceptible DNA samples from the NIL. Candidate primer pairs showing polymorphisms between the pooled R and S DNA samples were analyzed further using individual DNA samples isolated from the three pairs of NIL. Two AFLP primer combinations were identified to produce DNA fragments showing apparent differences between the susceptible and resistant lines. When primer pair B1 (E-AAG: 5′-GACTGCGTACCAATTCAAG; M-CAA: 5′-GATGAGTCCTGAGTAACAA) was used, a 378bp band was present in two resistant lines, M-120 RNR and M-249 RNR, but absent in their corresponding susceptible recurrent parents, Coker 201 and ST213. Line M-240 and its recurrent parent did not amplify the fragment (data not shown). Conversely, primer combination C3 (E-ACA: 5′-GACTGCGTACCAATTCACA; M-CAG: 5′-GATGAGTCCTGAGTAACAG) amplified a weak band of 412 bp (based on CEQ 8000 analysis on pooled samples) only present in the susceptible lines, but absent in all the resistant lines (data not shown). These results indicated that the polymorphic AFLP fragments generated by primers B1 and C3 are likely linked to the RKN resistance/susceptibility.

Using eight pairs of RGA primers, multiple PCR bands were amplified from the three pairs of RKN resistant and susceptible NIL. Polymorphic bands present in the resistant lines or susceptible lines were observed. The degenerated NBS primer pair (NBS-F1: 5-GGAATGGGNGGNGTNGGNAARAC; NBS-R1: 5-YCTAGTTGTRAYDATDAYYYTRC) produced a fragment of approximately 750bp, which was present only in the resistant lines. The degenerate primer pair RLK (RLK for: 5-GAYGTNAARCCIGARAA; RLK rev: 5-TCYGGYGCRATRTANCCNGGITGICC) amplified a minor polymorphic fragment of about 850bp in the susceptible lines. The degenerate primer pair Pto kin (Pto kin 1: 5-GCATTGGAACAAGGTGAA; Pto kin 2: 5-AGGGGGACCACCACGTAG) produced a fragment of ~460 bp in three susceptible lines and one resistant line M-120 (data not shown). These polymorphic bands were all very weak and could not be identified in agarose gel, but could be identified in high-resolution polyacrylamide gels.

Sixty 10-mer primers were also tested using the three pairs of NIL. Two RAPD primers were identified to consistently produce polymorphic bands between the RKN resistant lines and susceptible lines (Fig. 1). Primer UBC693 (5'-GACGAGACGG) amplified a polymorphic band (~420bp) from the three resistant NIL, M-120 RNR, M-240 RNR, and M-249 RNR. This fragment was absent in the respective susceptible recurrent parents (Fig. 1A). Primer UBC781 (5'-GGGAAGAAGG) amplified a polymorphic 1kb-band (964 bp) present only in the resistant lines (Fig. 1B). Sequence analysis revealed that these two polymorphic RAPD fragments had no significant sequence similarity, indicating that they were amplified from different loci.

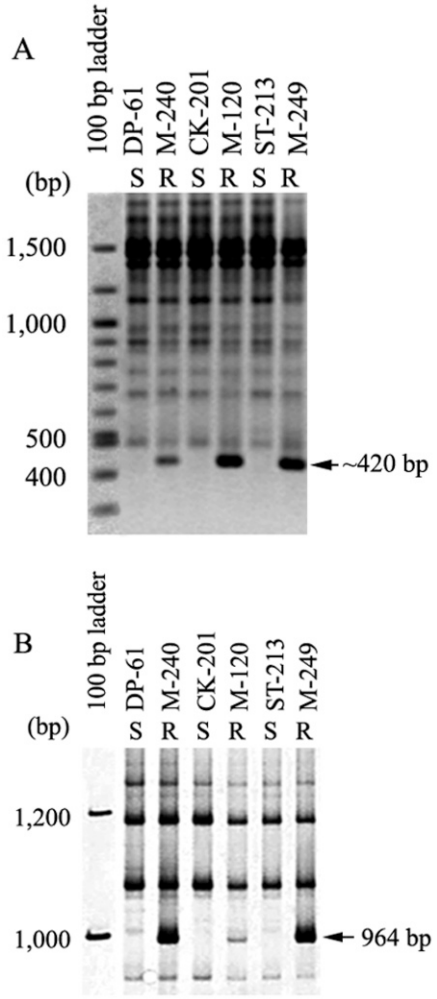

Figure 1. Generation of a DNA marker associated with cotton resistance to root-knot nematode. DNA samples prepared from three pairs of cotton NIL were subjected to RAPD analysis using the decamer primers UBC693 (A) and UBC781 (B). Products in (A) were separated on a 1.2% (w/v) agarose gel, stained with EtBr and products in (B) were separated on a 5% polyacrylamide gel and visualized by silver staining. The approximately 420-bp band in (A) and 1000-bp band in (B) are present only in the lanes representing the RKN resistant lines and indicated by the arrows.

Cloning, Sequencing, and STS Marker Conversion

To facilitate the application of marker-assisted screening and to characterize molecular markers, the potential RKN resistance-associated AFLP, RGA, and RAPD

polymorphic bands were cloned and sequenced. Based on sequence information, STS primers were designed for the above polymorphic AFLP, RGA, and RAPD bands. Although AFLP, RGA, and RAPD based UBC693STS primers produced fragments with expected sizes, no polymorphism was present in any of the three pairs of susceptible and resistant NIL. The UBC781-964STS primers (Forward: 5'-GGGAAGAAGGGGACGGGAGTTC; Reverse: 5'-GGGAAGAAGGCGATTGGTTGCAA), however, specifically amplified a 964-bp product designated as UBC781-964STS, which was present in the resistant lines and absent in the susceptible lines (Fig. 2). This STS marker was studied further with genomic DNA isolated from more germplasm lines. Similar to its original RAPD marker, the STS marker was absent in all the susceptible lines, but present in 12 resistant lines (Table 1). This result demonstrated that the RAPD marker UBC781-1000 was converted successfully into a reliable STS marker.

Marker Confirmation by Segregation Analysis

The RAPD marker UBC693-420 and the STS marker UBC781-964STS, together with the previously identified RKN resistance-associated SSR markers amplified by CIR316 and BNL1231 and one CAPS marker (GHACC1) (Wang et al., 2006b; Wang and Roberts, 2006), were tested in the F_2 population (90 plants) of ST 474 × Auburn 634 RNR. Figure 3 shows part of the tests. In this population, 49 plants were classified as resistant and 14 plants susceptible based on root-galling index. Twenty-seven plants died early due to poor growth and were treated as missing data in the mapping analysis, which gave similar results to that based on the 63 live plants with segregation conforming to a 3R:1S ratio ($\chi^2 = 0.259$, $P > 0.05$). This indicated that there is at least one major R gene involved in the RKN resistance detected in the segregating population, although the population size did not preclude other minor genes contributing to the resistance. McPherson et al. (2004) reported Mi_1 and Mi_2 in Auburn 634 RNR-derived M-315 RNR and Mi_1 in M-78 RNR which was derived from a race stock, T-78 RNR. Considering that (i) Wild Mexico Jack Jones was also a race stock which may also contain gene Mi_1; (ii) Clevewilt and its derived line ST LA 887 contain a recessive R gene which may be allelic to *rkn1* (Zhou, 1999; Bezawada et al., 2003); and (iii) the R gene is linked to the *rkn1*-associated markers (Shen et al., 2006; Wang and Roberts, 2006; Wang et al., 2006b; Ynturi et al., 2006; also see the following linkage analysis), we assign Mi_2 to the R gene detected by the segregation analysis.

The CAPS marker GHACC1 was resolved originally on agarose gel after restriction digestion of PCR product with enzyme *Nla*III (Wang and Roberts, 2006). On the high resolution polyacrylamide gel, undigested PCR products from resistant plants amplified with the same GHACC1 primers showed an extra fragment at approximately 320 bp compared with amplification from susceptible plants (Fig. 3C). The use of polyacrylamide gel eliminated the digestion procedure and this marker is hereafter named UCR-STS. All the markers displayed apparent segregations in a dominant fashion. For example, except for three failed amplifications, UBC781-964STS showed an expected 3:1 segregation ratio in the F_2 population (66 present: 21 absent). Linkage analysis revealed that UBC781-964STS was linked to a major RKN resistance gene, presumably Mi_2, with an estimated distance of 31.6 cM. UBC693-420 was located in the same linkage group being 39.6 cM from UBC781-964STS (Fig. 4). The closely linked CAPS marker GHACC1 (UCR-STS herein) and CIR316 (2.9 cM) were linked to the RKN resistance gene *rkn1* on cotton linkage group LG A03 (chromosome 11) with genetic distances of 0.8 and 2.1 cM, respectively, in an F_2 (NemX × SJ-2) segregating population (Wang and Roberts 2006; Wang et al., 2006b). These two markers were also closely linked (ca. 5.4 cM) in the F_2 of ST 474 × Auburn 634 RNR, and their estimated genetic distances to Mi_2 were 12.6 cM and 18.0 cM, respectively. The two markers, UCR-STS (23.7 cM) and CIR316 (18.3cM) were distantly linked to SSR marker BNL1231 in this population, similar to the data analyzed in the F_2 (NemX × SJ-2) segregating population (24.1cM and 26 cM, respectively) (Wang and Roberts 2006; Wang et al., 2006b). Therefore, the linkage analysis confirmed that (i) the two RAPD markers and one converted STS marker are indeed associated with a

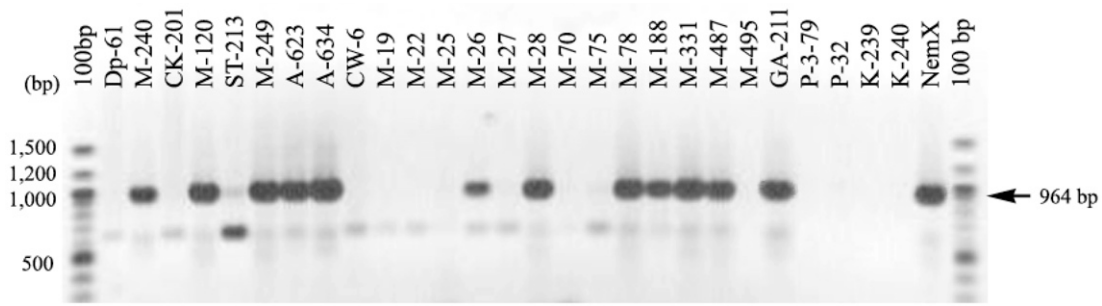

Figure 2. STS primers designed from the polymorphic 1000-bp amplification product generated by the RAPD primer UBC781 amplified a 964-bp UBC781 STS marker. The UBC781-STS marker is absent in all susceptible lines and present in some of the resistant lines.

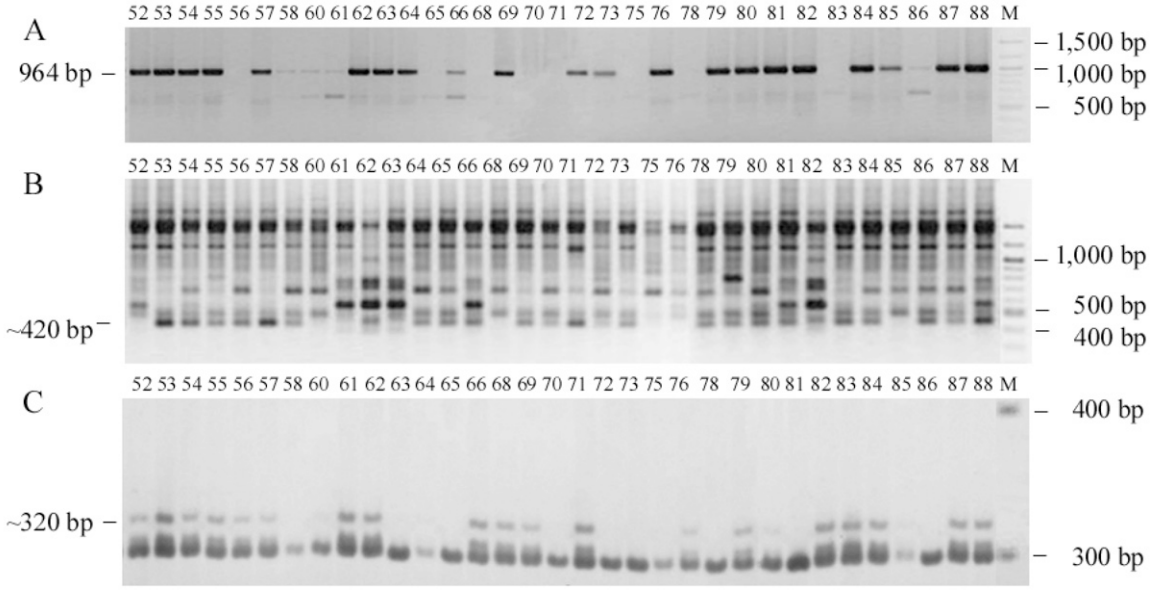

Figure 3. Gel image of amplification products representing the markers UBC781-STS (A), UBC693 (B), and UCR-STS (C) in part of an F_2 (ST 474 × Auburn 634) segregating population. The bands were resolved on agarose gels (A and B) and a polyacrylamide gel (C).

primary determinant of RKN resistance, presumably gene *Mi*$_2$, in Auburn 634 RNR; and (ii) they are also linked with the *rkn1* associated markers, CIR316, BNL1231, and GHACC1. Although our resistance-linked markers were not informative for use in the Acala NemX × Auburn 634 RNR F_2 population, resistance phenotyping revealed no susceptible recombinants among the 200 individuals. All galling indices were ≤ 3, with only 14 plants having a score of 3. By comparison, the mean (and range) of the parent and control plant galling indices were 0.3 (0–0.5) for Auburn 634 RNR, 1.7 (1–2.5) for Acala NemX, and 6.2 (5.5–7) for susceptible control Acala SJ-2.

Germplasm Survey Using RAPD, STS, CAPS, and SSR Markers

To test the feasibility of direct use of the above RAPD markers and to investigate the potential relationship of the RAPD markers with RKN resistance, UBC693-420 and UBC781-1000 were screened in 23 RKN resistant and 8 susceptible cotton cultivars or breeding lines (Table 1). The two markers were both present in Auburn 634 RNR and Auburn 623 RNR, as expected. UBC693-420 was present in 95.6% (22/23) resistant lines, while UBC781-1000 was present in 52.2% (12/23) resistant lines. These two markers were absent in all the susceptible lines, further confirming their association with RKN resistance. The converted STS marker UBC781-964STS showed the same results as its original RAPD marker (Table 1). Based on these RAPD and STS marker profiles, the tested germplasm could be classified into three groups (Table 1). In Group 1, all the Auburn 623 RNR-derived resistant lines contained the three markers. Interestingly, M-28 RNR and M-78 RNR were converted day-neutral lines from short-daylength race stocks, but they also contained the three markers, as did resistant Acala NemX, with an unknown source for resistance gene *rkn1*, and also GA96-211. The development of this germplasm involved the Auburn 623 RKN derived resistant lines M-240 RNR and M-120 RNR in its pedigree (May et al., 2004). In Group 2, most of the race stock converted resistant lines and Wild Mexican Jack Jones contained UBC693-420. In Group 3, none of the susceptible lines had the two markers. Unexpectedly, Clevewilt 6 contained none of the three markers.

The UCR-STS (CAPS) and CIR316 markers associated with *rkn1* in Acala NemX were tested with additional germplasm (Table 1). The UCR-STS marker was present in all highly or moderately resistant breeding lines, and was present in all susceptible *G. barbadense* germplasm

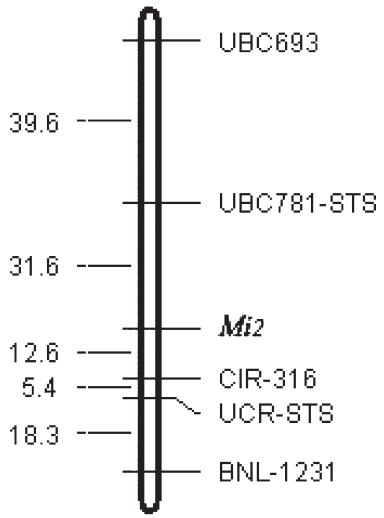

Figure 4. Location of UBC781-STS and UBC693 markers relative to the RKN resistance gene *Mi*$_2$ and resistance markers reported in other studies. The linkage map was generated from an F_2 (ST 474 × Auburn 634 RNR) segregating population.

lines (Table 1). The CIR316 marker was present in 15 of 23 R lines and absent in all the S lines (Table 1).

DISCUSSION

Evaluation of RKN infestation on cotton is a very costly, labor intensive, and time consuming process. Molecular markers closely associated with one or more nematode resistance genes could be used as reliable and accurate tools for RKN resistance screening, which may minimize the difficulties associated with field and greenhouse resistance phenotype evaluations. Recently a few groups reported the development of molecular markers targeted to cotton RKN resistance using different resistant germplasm (Bezawada et al., 2003; Shen et al., 2006; Wang et al., 2006b; Wang and Roberts, 2006; Ynturi et al., 2006; Zhang et al., 2006b). One SSR marker (CIR316) and one CAPS marker (GHACC1) were mapped to cotton chromosome 11 (formerly linkage group LG A03) with genetic distance less than 3 cM to a major RKN resistance gene (*rkn1*) in Acala NemX derived populations (Wang and Roberts, 2006; Wang et al., 2006b). The presence of multiple RKN resistance associated loci and polymorphic behavior of RKN associated markers among R breeding lines, such as the behavior of CIR316 and UCR-STS in Table 1, implies that additional molecular markers need to be identified for efficient application in a wide range of cotton germplasm. In the present study, these markers linked to *rkn1* have been confirmed to be associated with the RKN resistance derived from Auburn 634 RNR, presumably gene Mi_2. In addition, we have identified and confirmed two new RAPD markers that are linked to the Mi_2 gene. The UBC693-420 and UBC781-964STS markers were mapped to the same linkage group with Mi_2 and several markers previously found linked with the RKN resistance gene *rkn1* in Acala NemX (Wang et al., 2006b; Wang and Roberts, 2006), and RKN R gene or QTL in the Auburn resistance source (Shen et al., 2006; Ynturi et al., 2006), validating their potential usefulness in MAS for both the Auburn 634 RNR and Acala NemX resistance sources. The successfully converted UBC781-964STS is easily visualized on an agarose gel and may be a useful RKN resistance marker in both cultivated allotetraploid species of cotton, *G. hirsutum* and *G. barbadense*. In a separate study, Shen et al. (2006) confirmed the linkage of CIR316 to nematode resistance in *G. hirsutum*, using an M-120 source derived from Auburn 623 RNR. Compared with phenotyping evaluation, the molecular marker systems reported for RKN resistance in cotton can achieve objective selection and minimize laborious greenhouse inoculation and screening. Among the marker systems, AFLP, RAPD, and SSR analysis usually show multiple bands on polyacryamide or agarose gels and are suitable for initial marker selection, while STS and CAP markers usually show simple and more specific band patterns and are easily observed on inexpensive agarose gels.

Though the genetic distance of UCR-STS and CIR 316 to Mi_2 in the Auburn 634 RNR population is larger than their distance to *rkn1* in the NemX population (Wang and Roberts, 2006), > 12.6 cM vs. < 3 cM, it is likely that *rkn1* and Mi_2 are either allelic or closely linked. The absence of any susceptible recombinants in the Acala NemX × Auburn 634 RNR-derived F_2 population further supports this association between *rkn1* and Mi_2. Even though the resistance source for Acala NemX is not definitive, it may share a similar resistance source to Auburn 623 RNR. Both Mi_2 and *rkn1* are on the same chromosome and the three markers linked to *rkn1* were also found to be polymorphic between resistant lines of Auburn source and susceptible lines, and monomorphic in susceptible genotypes. These three markers were also polymorphic in the ST 474 × Auburn 634 RNR F_2 population and linked to one another with similar genetic distances as reported for the Acala NemX population (Wang and Roberts, 2006; Wang et al., 2006b). The markers are also linked to gene Mi_2, but population type and variation in resistance screening could result in differences in the estimated genetic distances between the markers and RKN resistance genes (*rkn1* or Mi_2). It is interesting to note that these markers were present in highly RKN resistant breeding lines Auburn 623 RNR, Auburn 634 RNR, and their derived lines (M-188 RNR, M-331 RNR, and M-487 RNR) (Shepherd et al., 1989), as well as in Acala NemX. The CIR316 and UCR-CAPS markers present in many resistant M-lines supported further the postulation that Auburn 623 RNR and Acala NemX either carry the same resistance gene(s) or, since they show dominant and recessive resistance inheritance behavior respectively, more likely have linked but different resistance genes clustered on chromosome 11. It should be pointed out that the estimated genetic distances from UBC693-420 and UBC781-964STS to Mi_2 in the linkage map (Fig. 4) were large and better resolution of these genetic distances will require further evaluation and allelism tests between *rkn1* and Mi_2. It also should be pointed out that results on the inheritance of the RKN resistance trait have lead to different conclusions, with an incomplete dominance mode in Auburn 623 RNR initially reported by Shepherd (1974a). Through classical genetics, McPherson et al. (2004) reported the presence of one dominant gene and one additive gene in M-315 RNR derived from Auburn 623 RNR, while a major dominant gene on chromosome 11 and a minor dominant gene on chromosome 7 in M-120 RNR (also derived from Auburn 623 RNR) were detected through QTL mapping (Shen et al., 2006). Furthermore, Shen et al. (2006) reported that the major dominant gene in M-120 RNR is coincidently located in the same region on chromosome 11 as recessive gene *rkn1* in Acala NemX. However, more recently, Ynturi et al. (2006) confirmed that the R gene on chromosome

11 as identified by BNL1231 is additive with no dominant effect, while another R gene on chromosome 14 had dominant and additive effect. Therefore, our study agrees with the most recent reports in that one R gene is located on chromosome 11 as tagged by the SSR marker BNL1231 and three new markers in the Auburn source and NemX. A single gene, *rkn1*, with recessive behavior in *G. hirsutum* crosses primarily determines RKN resistance in Acala NemX (Zhou et al., 1999; Wang et al., 2006b). However, it remains to be resolved (i) whether Mi_2 and *rkn1* are allelic or linked; and (ii) where the second R gene, Mi_1 is located. Further studies including large allelism tests are required to resolve these issues.

Sequence-tagged site (STS) markers have more advantages and fewer disadvantages than other marker types. In an attempt to obtain more specific amplification of molecular markers, polymorphic bands from AFLP, RGA, and RAPD were cloned and sequenced. However, most of these AFLP, RGA, and RAPD based STS markers lost their original polymorphisms. Such phenomena were encountered in other studies (Meksem et al., 2001; Xu and Ban 2004; Hinchliffe et al., 2005). The major reasons could be band contamination, or multiple sequences (Meksem et al., 2001; Xu and Ban 2004; Hinchliffe et al., 2005). The use of polyacrylamide gels may facilitate the isolation of bands that represent only one DNA sequence, and subsequently facilitate conversion to polymorphic STS markers. However, Meksem et al. (2001) reported an average of six sequences per AFLP band, indicating that more cloning and sequence information might be required to convert the AFLP, UBC693-420, and RGA bands into useful STS markers.

Our interest is in developing markers associated with RKN resistance for MAS and identification of gene(s) directly involved in RKN resistance in cultivated cotton. In working with two different lines derived from the Auburn source, it was suggested that the high level of RKN resistance in these lines was controlled by only two major genes (Zhou et al., 1999; McPherson et al., 2004; Zhang et al., 2004). The two RAPD markers and one STS marker identified here, together with the three markers linked to *rkn1*, support that the RKN resistance in the Auburn source has a primary determinant located on chromosome 11 (formerly LG A03). However, our results with a fairly small mapping population do not preclude the involvement of additional minor gene(s) contributing to resistance, as indicated by these other inheritance studies and most recently by QTL mapping (Shen et al., 2006; Ynturi et al., 2006). Molecular mapping using the current and other markers will help clarify the genetic basis of nematode resistance in cotton. The markers studied here were present only in the resistant lines and absent in susceptible lines, demonstrating their utility in MAS. The RKN resistance-associated DNA markers UBC693-420 and UBC781-964STS identified in this study show obvious polymorphism between resistant M-lines and their recurrent parents, and can be grouped with known molecular markers closely associated with the *rkn1* gene in Acala NemX. This close association indicates their potential usefulness in RKN resistant cotton breeding and map-based cloning of RKN resistance genes. The CAPS, STS, and SSR markers provide a relatively quick and efficient resistance selection system, compared with RAPD, RFLP, and AFLP markers, and with resistance phenotyping.

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